ISSN 1392-3196
Žemdirbystė=Agriculture, vol. 97, No. 4 (2010), p. 91–98
UDK 633.16“321”:632.482

Pyrenophora teres genetic diversity as detected by ISSR analysis

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Abstract

Specific PCR and inter simple sequence repeat (ISSR) marker systems were used to study genetic diversity in barley pathogen *Pyrenophora teres* isolates collected in 5 districts of Lithuania during 2006–2009. Specific PCR was used to determine the net/spot form and mating type occurrence in net blotch field populations. The occurrence of mating types and net/spot were investigated in all years/locations, however only *Pyrenophora teres* f. *teres* was detected in all investigated populations. Both mating types were found and their ratio was 1:1 in all populations except for Klaipėda distr. 2008. Both mating types were found in Klaipėda population as well, but the observed ratio did not meet the expected. A total of 24 isolates from Kėdainiai and Marijampolė distr. 2007–2008 were selected for ISSR analysis. Primers UBC807 and G11 were most suitable. The selected primers amplified 31 bands in total within the range of 500 bp to 2500 bp and all isolates had unique ISSR haplotypes. Analysis of molecular variance (AMOVA) detected most of the variation within populations – 88.78%, while the variation between populations from the same location in different years accounted for 6.37%, and the variation between different locations in the same year was the smallest – 4.85% of the total variation. No clear grouping according to location or year of sampling was detected in cluster analysis.

Key words: net blotch, *Pyrenophora teres*, ISSR, genetic diversity, mating types, barley, *Hordeum vulgare*.

Introduction

Pyrenophora teres Drechs. is the causal agent of barley net blotch. Its imperfect stage is *Drechslera teres* (Sacc.) Shoem. It comprises two types. The net type *P. teres* f. *teres* produces horizontal and vertical crisscrossed dark brown venation on the barley leaves that can turn chlorotic. The spot type *P. teres* f. *maculata* causes dark brown circular or elliptical spots surrounded by chlorosis (Smedegard-Petersen, 1977). Both types can cause significant financial losses due to yield reductions, ranging from 15 to 35%, and decreased grain quality (Steffenson et al., 1991). Fungicides under high net blotch pressure can save 18–50% grain yield in Lithuanian conditions (Semaškienė et al., 2009). The conidia of these two types do not differ morphologically. The resistance to spot and net types is inherited independently (Scott, 1992; Arabi et al., 2003). The fungus can survive on stubble over winter, and reproduces asexually during growing season (Duczek et al., 1999). Sexual reproduction is also possible. The net blotch fungus is heterothallic and self-sterile (McDonald, 1963), and in heterothallic fungi, the sexual cycle is initiated only when two fungal strains of different mating types interact, after each detects the other through mating pheromones produced by the unlike mating type (Kronstad, Staben, 1997). Mating type surveys can be used to gauge the potential of sexual recombination.

The durability of host resistance to pathogenic fungus and also the effectiveness of fungicides depend on the ability of the pathogen population to evolve so that it can overcome resistance, and pathogens with a high evolutionary potential are more likely to do that (McDonald, Linde, 2002). Mutation plays important role in enlarging *P. teres* genetic diversity, because it is haploid for the most

time of its life cycle. Net blotch can spread through large territories through the infected seed. Moreover, net blotch can reproduce both sexually and asexually, so it has a big evolutionary potential and its population comprises a large number of genotypes and pathotypes. In a preliminary study by Peltonen et al. (1996), no genetic differentiation between Finnish net blotch isolates according to location, aggressiveness, growth rate or morphological features was detected. However, genetic structure of populations could not be studies due to small number of isolates from the same location. In a later study most of the genetic variation was observed within field population and no genetic differentiation between mating types within population (Serenius et al., 2005). Large net blotch genetic diversity was detected in Czechia (Lei ova et al., 2005 a, b), Sweden (Jonsson et al., 2000), North America (Peever, Milgroom, 1994), South Africa and Australia (Lehmensiek et al., 2010). The information about the reproduction system and genetic structure of pathogen population is useful in planning plant breeding programmes and fungicide control strategies.

Inter simple sequence repeat (ISSR) marker system is PCR-based DNA fingerprinting technique. DNA segments, situated between inverted microsatellites are amplified; therefore no information about DNA sequence is required. ISSR was first described by Zietkiewicz et al. (1994). It was primarily used for plant and animal population genetic studies, but it was also successfully applied for fungi (Borja et al., 2006; Armengol et al., 2010). The genetic diversity of *P. teres* has been investigated mostly by using RAPD and AFLP markers. ISSR markers were used for wheat tan spot *Pyrenophora tritici-repentis*, which belongs to the same genus as barley net blotch *P. teres*, genetic diversity studies (Moreno et al., 2008), but we did not find any studies using ISSR technique to investigate *P. teres*.

The objective of this study was to investigate barley net blotch *P. teres* genetic diversity by using ISSR markers and the occurrence of *P. teres* f. *teres* / *P. teres* f. *maculata* and MAT1 / MAT2 mating types in field populations in Lithuania.

Materials and methods

The occurrence of mating types and net and spot forms of net blotch in field populations was investigated during 2006–2009, in 5 different districts of Lithuania (Table 1).

Table 1. The sites of spring barley, infected with net blotch, leaf sample collection

District	Coordinate	Year of collection	No of isolates in net/spot PCR	No of isolates in mating type PCR
Kėdainiai	N 55º 23′ L 23 º 52′	2006	34	24
		2007	35	26
		2008	49	49
		2009	19	19
Marijampolė	N 54º 28′ L 23º 20′	2007	12	12
		2008	33	23
Ignalina	N 55º 26′ L 26º 8′	2007	12	12
		2008	35	35
		2009	29	29
Biržai	N 56º 11′ L 24º 48′	2008	32	32
Klaipėda	N 55º 47′ L 21º 8′	2008	32	32

Fungal isolates. Naturally infected spring barley leaves showing typical net blotch symptoms were collected during crop season at milk growth stage. The dry leaves were kept in paper bags in dry conditions until the cultivation of fungus. Surface-sterilized leaf sections were placed on moist filter paper in Petri dishes and incubated at 20°C for 4 days, 12 h light / 12 h dark. Single conidia were transferred on 1.5% V8 agar and incubated for 10 days at the same conditions.

DNA isolation. Small sections of mycelium were transferred to liquid V8 media and incubated on a rotating shaker at 100 rpm, 20°C for 4–5 days. Media was poured away; mycelium was washed with distilled water. DNA was isolated according to modified protocol of Lassner et al. (1989).

PCR conditions for net/spot forms. Amplification was performed according to Williams et al. (2001). PCR was carried out in 15 µl reaction mixture, containing 1 x DreamTaq PCR buffer ("Fer-

mentas", Lithuania) 1.5 mM $MgCl_2$, 1 µM of each primer (MAT, PTT-R, PTM-F and PTM-R; "Metabion", Germany), 0.2 mM dNTP, 0.8u DreamTaq polymerase ("Fermentas", Lithuania), 25 ng of DNA. PCR thermal profile: 94°C – 1 min, 10 cycles 94°C – 30 s, 65°C (reducing 1°C per cycle) – 30 s, 72°C – 1 min, followed by 24 cycles 94°C – 30 s, 55°C – 30 s, 72°C – 1 min. Reaction was ended with 5 min extension at 72°C.

PCR conditions for mating types. Amplification was performed according to Rau et al. (2005). PCR was carried out in 20 µl reaction mixture, containing 1 x DreamTaq PCR buffer ("Fermentas", Lithuania) 2.5 mM $MgCl_2$, 1 µM of each primer (MAT1-F, MAT1-R, MAT2-F, MAT2-R; "Metabion", Germany), 0.2 mM dNTP, 1 u DreamTaq polymerase ("Fermentas", Lithuania), 25 ng of DNA. PCR thermal profile: 94°C – 1 min, followed by 30 cycles 94°C – 60 s, 55°C – 60 s, 72°C – 90 s. Reaction was ended with 10 min extension at 72°C.

ISSR analysis. Twelve microsatellite primers ("Metabion", Germany) were tested for ISSR analysis, and two of them were selected for genetic diversity investigation. PCR was carried out in 15 µl, containing 50 ng DNA, 1 x DreamTaq PCR buffer, 1.87 mM $MgCl_2$, 0,2 mM dNTP, 0.25 µM of primer and 0.5 u DreamTaq polymerase ("Fermentas", Lithuania). PCR thermal profile: 95ºC – 2 min, 40 cycles 95ºC – 30 s, 50ºC – 60 s, 72ºC – 60 s, and 6 min 72°C at the end.

All PCR reactions were carried out in Mastercycler thermocycler ("Eppendorf", Germany).

Scoring and data analysis. Amplification products were separated by electrophoresis on 1.5% agarose gel containing ethidium bromide. The gels were run in 1 x TAE buffer for 1.5 h at 100 V. The separated fragments were photographed in UV light. The sizes of fragments were measured by comparing them to GeneRuler™ DNA Ladder Mix ("Fermentas", Lithuania).

Standard diversity indices and AMOVA analysis were calculated with *Arlequin ver 3.0* software (Excoffier et al., 2005). NTSYSpc v. 2.2 (*Exeter Software*, "Setauket", NY) (Rohlf, 2005) was used for computing similarity indexes and dendrogram drawing. The mating type ratio was tested with a χ^2 significance test.

Results and discussions

Net/spot form and mating type occurrence. Mating types and net/spot types were analysed using specific PCR. Isolate SIR1S from Italy was used as spot form control (Figure 1). All isolates, in all locations and all years of investigation, were net type *P. teres* f. *teres*.

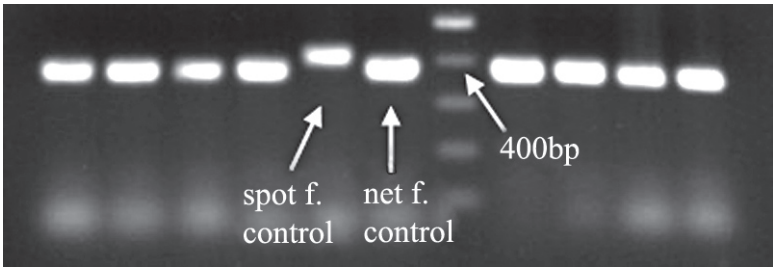

Figure 1. Net/spot type specific PCR electrophoresis – net type primers amplified 378 bp band, spot type primers – 411 bp

The occurrence of spot/net types is variable among countries in Europe and elsewhere. Net type is more common in Scandinavia (Serenius et al., 2005), but both types are common in Italy (Rau et al., 2003). Spot type was recently detected in Hungary for the first time (Fiscor et al., 2010). Spot form dominates in Australia (McLean et al., 2009). The resistance to spot and net types is inherited independently, therefore it is possible to make a presumption that the occurrence of these types and the changes in their prevalence could be related to the popularity of certain barley varieties in different regions, yet the exact reasons remain unclear.

Asexual reproduction spores – conidia, are more important for the spread of net blotch, since conidia are formed during all growing season, while ascospores are produced only once, at the beginning of the season, but sexual reproduction may be important for the formation of new pathotypes. *P. teres* is self-sterile, and both MAT1 and MAT2 mating types are required for sexual reproduction to take place. Both mating types were found in all populations that were investigated (Figure 2).

The ratio of mating types was not significantly different from 1:1 ratio in most of the cases (Table 2). However, in Klaipėda district *P* was less than 0.5. In other studies the ratio became 1:1 when more isolates were added to analysis (Serenius et al., 2005), so it is likely that this result was obtained because of sampling bias. Our data coincide with the

results of other researches – in most cases the two mating types are found in 1:1 ratio (Rau et al., 2005; Serenius et al., 2005; Serenius et al., 2007), except for Russia, Krasnodar district, where only MAT-2 mating type was found (Serenius et al., 2007).

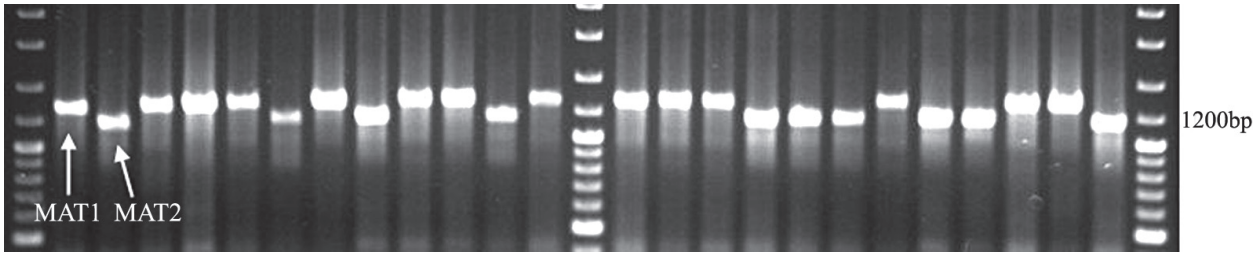

Figure 2. Mating type specific PCR electrophoresis – MAT1 primers amplified 1300 bp band, MAT2 primers – 1150 bp

Table 2. The ratio of mating types

District	Year	MAT1	MAT2	χ^2 (1:1)	$P^*_{\chi^2}$
Kėdainiai	2006	10	14	0.67	0.41
	2007	12	14	0.15	0.70
	2008	23	26	0.18	0.67
	2009	12	7	1.32	0.25
Marijampolė	2007	6	6	0	1.00
	2008	13	20	1.48	0.22
Ignalina	2007	5	7	0.69	0.19
	2008	15	20	0.71	0.40
	2009	12	17	0.86	0.35
Biržai	2008	18	14	0.50	0.48
Klaipėda	2008	7	25	10.13	0

Note. * – df = 1.

The presence of both mating types in close proximity makes mating possible, although that does not necessarily mean sexual reproduction is taking place. The ratio 1:1 is expected when segregation distortion and clonal selection among mating types are absent or asexual reproduction is rare (Rau et al., 2005), yet the latter possibility seems hardly possible, since asexual reproduction by conidia plays a major role in the spread of infection during barley growing season.

ISSR analysis. Twenty-four net blotch isolates were selected for ISSR analysis: 6 from Kėdainiai district 2007, 6 from Kėdainiai 2008 and the same amount of isolates from Marijampolė district 2007 and 2008. Twelve primers were tested (Table 3). Two primers – UBC807 (Figure 3) and G11 (Figure 4), were selected as the most suitable. They amplified the largest numbers of clear bands. Only those bands that reproduced at least three times were scored. The selected primers amplified 31 bands in total within range of 500 bp to 2500 bp. All isolates had unique banding patterns. Two isolates generated ISSR haplotypes that differed only in one band. They both were from Kėdainiai district 2008, mating type MAT2. In a *P. tritici-repentis* genetic diversity study using ISSR analysis, 35 out of 37 isolates had unique haplotypes (Moreno et al., 2008), indicating that both fungi populations are very heterogeneous.

Analysis of molecular variance (AMOVA) detected most of the variation within populations – 88.78%, while the variation between populations from the same location in different years accounted for 6.37%, and the variation between different sampling locations was smallest – 4.85% of total variation (Table 4). Similar results were obtained in studies of populations of other ascomycetes *Fusarium poae* (Dinolfo et al., 2010), *F. gramineum* (Mishra et al., 2004). When AFLP markers were used for net blotch genetic diversity investigation, the percentage of variation was also largest within populations (Serenius et al., 2005).

Table 3. The ISSR primers, tested for suitability for net blotch genetic diversity evaluation

Primer	Nucleotide sequence	Annealing temperature ºC*	Quality of PCR product
UBC807	$(AG)_8T$	45	++
UBC810	$(TGA)_8T$	45	+
UBC811	$(AG)_8C$	48	+
UBC814	$(CT)_8A$	45	+
UBC820	$(GT)_8C$	48	−
UBC822	$(TC)_8A$	45	+
UBC825	$(AC)_8T$	45	+
UBC826	$(AC)_8C$	48	−
UBC827	$(AC)_8G$	48	+
UBC866	(CTC)6	50	+
105H	$(GA)_8CT$	48	+
G11	$(CAA)_5GC$	45	++

Note. − no product, + too small number of bands or/and poor band intensity, ++ optimal band number, good band intensity and reproducibility.

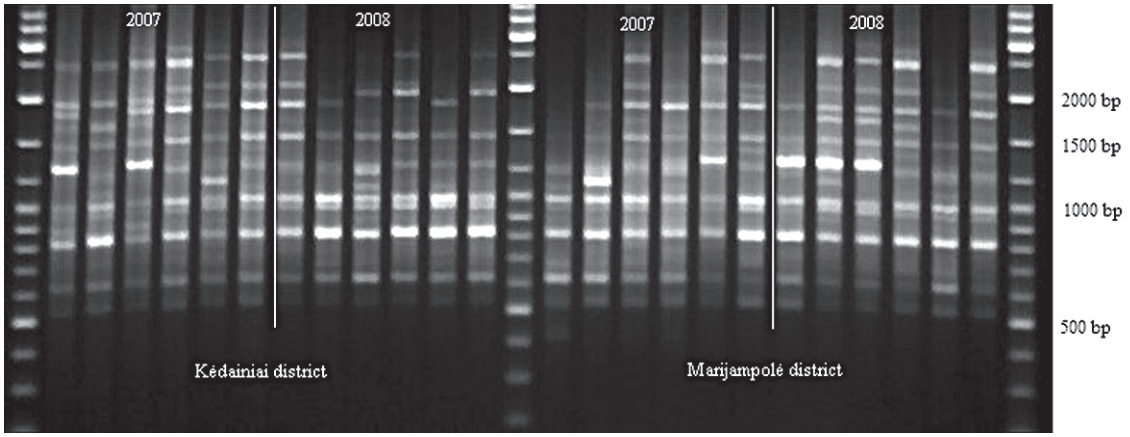

Figure 3. ISSR fingerprints of net blotch isolates produced by primer UBC807

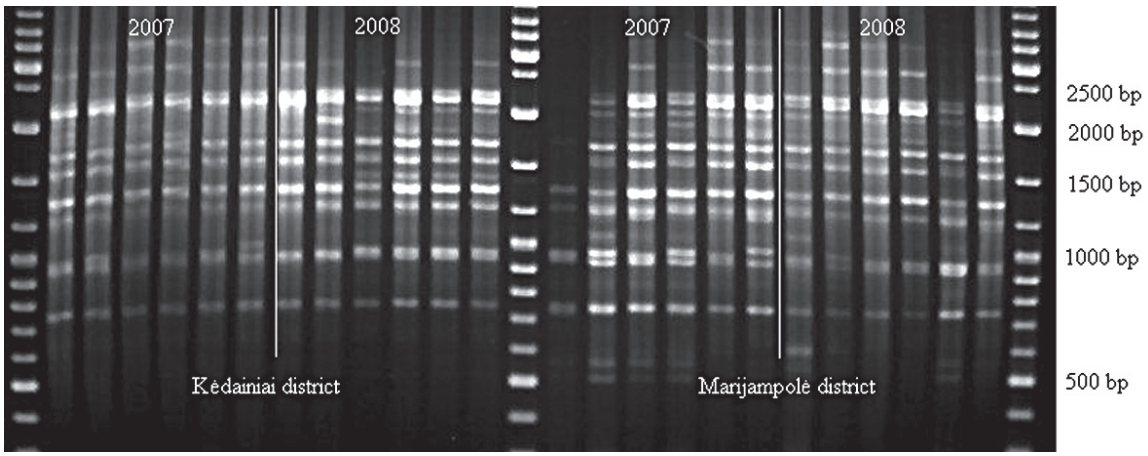

Figure 4. ISSR fingerprints of net blotch isolates produced by primer G11

Table 4. Analysis of molecular variance (AMOVA) of ISSR haplotypes

Source of variation	Degrees of freedom	Variance components	Percentage of variation
Among groups	1	0.17361	4.85
Among populations within groups	2	0.22778	6.37
Within populations	20	3.17500	88.78
Total	23	3.57639	100

Note. The first number in the name of each isolate indicates the year of sampling, letters K and M stand for Kėdainiai and Marijampolė, respectively.

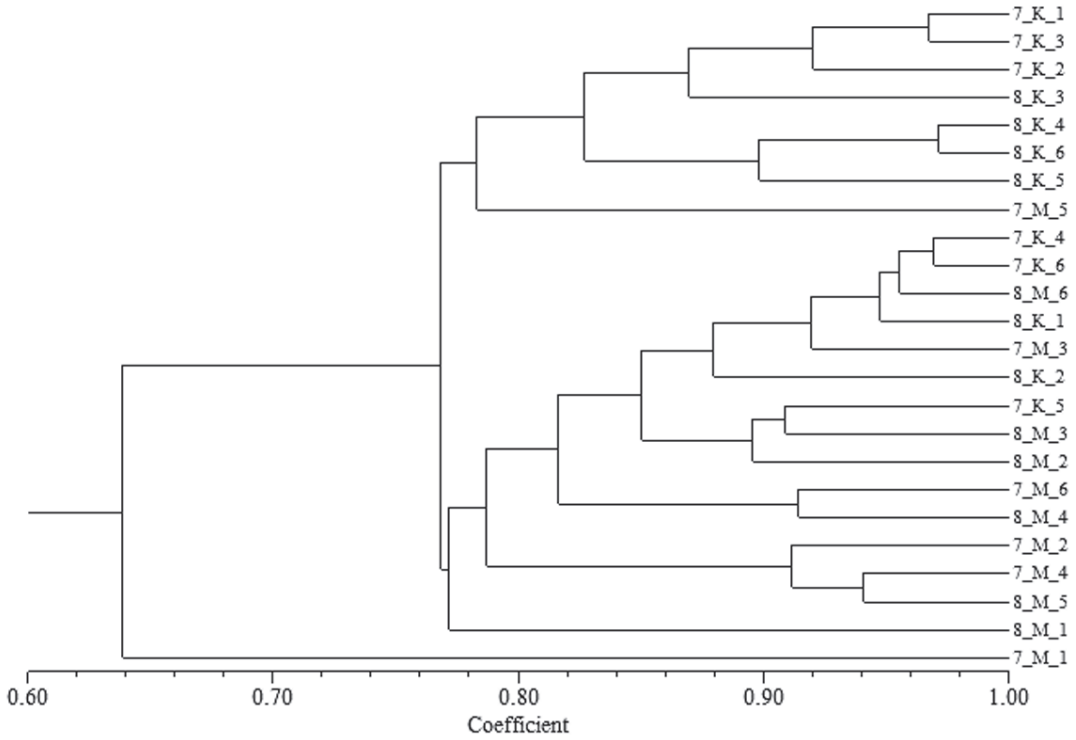

Figure 5. Dendrogram of net blotch isolates, constructed using UPGMA algorithm based on DICE similarity coefficient

The dendrogram was constructed using UPGMA algorithm based on DICE similarity coefficient. There are two main blocks in the dendrogram. Three isolates from Kėdainiai distr. 2007, mating type MAT1, and 3 isolates from Kėdainiai distr. 2008, mating type MAT2, fall into separate groups in the first block. But the DICE similarity coefficient is high at the nodes of both groups, 0.93 and 0.9 respectively. The rest of the isolates do not make distinct groups according to location, year of sampling or mating type. This was in agreement with AMOVA analysis – no differentiation between locations or sampling years, most of the variation is detected within populations.

In most cases of *P. teres* population structure investigation the largest percentage of variation is found within field population (Jonnson et al., 2000; Rau et al., 2003; Serenius et al., 2005; Lehmensiek et al., 2010). Variation between sampling locations is variable depending on many factors, including the distance between locations and also *P. teres* form – genetic divergence is higher between *P. teres* f. *teres* populations than *P. teres* f. *maculata* (Rau et al., 2003; Serenius et al., 2007; Lehmensiek et al., 2010). In this study only net form isolates were used, and the distance between sampling locations was 110 km. In a study by Jonsson et al. (2000) the distance between sampling sites was 20 km, but the variation between populations was similar – 5.3%. Serenius et al. (2005) studied genetic structure of two Finnish *P. teres* f. *teres* populations using AFLP markers. The distance between locations was 400 km, and variation between locations was 30.3%. In a study by Lehmensiek et al. (2010) similar variation was observed between *P. teres* f. *teres* populations in South Africa (32.81%), but only 8.89%

in Australia. Lei ova et al. (2005 b) predicates that variability seems to be more influenced by the year of sampling than the geographic origin of the isolate. In this study variation between different years of sampling was also slightly higher than variation between locations.

Conclusions

1. Only *Pyrenophora teres* f. *teres* was detected in all investigated populations.

2. Both mating types were found. Their ratio was 1:1 in all populations except for Klaipėda distr. 2008. Both mating types were found in Klaipėda population as well, but the ratio was not 1:1.

3. Primers UBC807 and G11 were the most suitable for net blotch ISSR analysis. The selected primers amplified 31 bands within the range of 500 bp to 2500 bp. All isolates had unique ISSR haplotypes.

4. Analysis of molecular variance (AMOVA) detected most of the variation within populations – 88.78%, while the variation between populations from the same location in different years accounted for 6.37%, and the variation between different locations in the same year was smallest – 4.85% of total variation. No clear grouping according to location, year of sampling or mating type was detected in cluster analysis.

Acknowledgements

The present study was supported by the Lithuanian State Science and Studies Foundation (2008–2009) and Research Council of Lithuania (2010), project GENŪKIS.

Received 19 10 2010
Accepted 01 12 2010

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ISSN 1392-3196
Žemdirbystė=Agriculture, vol. 97, No. 4 (2010), p. 91–98
UDK 633.16“321”:632.482

Pyrenophora teres genetinės įvairovės tyrimai naudojant ISSR žymeklius

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Santrauka

Paprastųjų pasikartojančių sekų intarpų (ISSR) žymekliai buvo panaudoti tiriant tinkliškosios dryžligės sukėlėjo *Pyrenophora teres* genetinę įvairovę. Lytinio dauginimosi tipai ir tinklo / dėmių forma nustatyta PGR metodu su specifiniais pradmenimis. *Pyrenophora teres* lytinio dauginimosi tipų ir formų paplitimas 2006–2009 m. tirtas 5 Lietuvos rajonuose. Visose tirtose populiacijose aptikta tik tinklo forma *P. teres* f. *teres*. Nustatyti abu lytinio dauginimosi tipai MAT1 ir MAT2, jų santykis buvo 1:1 visose populiacijose, išskyrus 2008 m. aptiktus Klaipėdos r. ISSR analizei pasirinkti 24 izoliatai iš Kėdainių ir Marijampolės r. 2007–2008 m. ištirtų populiacijų. Panaudota 12 ISSR pradmenų, du iš jų (UBC807 ir G11) atrinkti kaip tinkamiausi. Pasirinkti pradmenys amplifikavo 31 fragmentą, jų dydžiai pasiskirstė nuo 500 iki 2 500 bp. Visų izoliatų DNR antspaudai buvo skirtingi. Atlikus AMOVA analizę nustatyta, kad didžiausias variacijos procentas buvo populiacijų viduje – 88,78 %, variacija tarp populiacijų iš tos pačios vietovės, bet skirtingų metų buvo 6,37 %, variacija tarp tų pačių metų skirtingų vietovių populiacijų – 4,85 %. Atlikus klasterinę analizę grupavimasis pagal vietovę, tyrimų metus ar lytinio dauginimosi tipą nenustatytas.

Reikšminiai žodžiai: tinkliškoji dryžligė, *Pyrenophora teres*, ISSR, genetinė įvairovė, lytinio dauginimosi tipai, vasariniai miežiai, *Hordeum vulgare*.